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Susceptibility Studies of Multiply Resistant *Haemophilus influenzae* Isolated from Pediatric Patients and Contacts

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From February 1981 to December 1983, 225 strains were isolated from pediatric patients infected with *Haemophilus influenzae*. Forty-one strains were found to be resistant to ampicillin, chloramphenicol, and other antibiotics. They were isolated from 20 patients with invasive diseases (meningitis, 16; bacteremia, 4) and 21 with noninvasive diseases (otitis media, 19; conjunctivitis, 2). During this period, 44 patients with invasive diseases were seen (meningitis, 28; bacteremia, 16). Strains resistant to both ampicillin and chloramphenicol occurred in 45.4% of cerebrospinal fluid and blood isolates and in 51% of cerebrospinal fluid isolates only. In this group, individual resistance to ampicillin was 50%; chloramphenicol, 52.2%; tetracycline, 54.5%; and sulfamethoxazole-trimethoprim, 63.6%. No epidemiological relationship could be found among the patients. The presence of asymptomatic carriers was investigated in two nurseries and in eight family groups. From a total of 125 individuals studied, 80 were found to be colonized by *H. influenzae*, and 36 carried multiply resistant strains. From patients and carriers, 77 strains were found to be resistant to ampicillin, chloramphenicol, and other drugs; 39 belonged to type b (cerebrospinal fluid, 16; blood, 4; ear, 7; and nasopharynx, 12), and 38 were non-type b. The most frequent pattern of resistance was ampicillin-chloramphenicol-tetracycline-sulfamethoxazole-trimethoprim (94.8%), followed by ampicillin-chloramphenicol-tetracycline (3.9%). The disk diffusion method correctly predicted multiple resistance. The mean inhibition zone diameters were: ampicillin, 12.8 mm; chloramphenicol, 15.2 mm; tetracycline, 9.9 mm; and sulfamethoxazole-trimethoprim, 10.8 mm. These resistant strains were susceptible to cefotaxime, moxalactam, cefoperazone, cefuroxime, rifampin, and gentamicin. Our data suggest that in Spain the resistance of *H. influenzae* to ampicillin and chloramphenicol is endemic and that other effective therapeutic modalities are needed.

Ampicillin-resistant isolates of *Haemophilus influenzae* type b were first recognized in 1974 (2, 3). Chloramphenicol continues to be the most widely accepted alternative to ampicillin, but occasionally chloramphenicol-resistant organisms have been found (4, 5). Since 1980, there have been occasional reports of strains of *H. influenzae* resistant to ampicillin and chloramphenicol in the United States (9, 19), Thailand (16), and England (8, 13), although no country has indicated that this problem constitutes an emergency situation. We recently reported (1a) a high prevalence of ampicillin- and chloramphenicol-resistant *H. influenzae* strains among our clinical isolates.

This work summarizes the susceptibility studies performed on 77 multiply resistant *H. influenzae* strains isolated between February 1981 and December 1983, both from patients and from their contacts at the Hospital Infantil San Juan de Dios in Barcelona, Spain.

MATERIALS AND METHODS

Bacterial strains. A total of 225 strains of *H. influenzae* were isolated from unrelated pediatric patients during the study period. The sources (with number of isolates) included cerebrospinal fluid (CSF), 28; blood, 16; middle-ear effusions, 138; bronchial aspirates, 25; and miscellaneous sources, 18.

Also, we carried out a study of carriers in two different nurseries in which there were two children that had had meningitis caused by strains of *H. influenzae* resistant to both ampicillin and chloramphenicol. Nasopharyngeal samples from 103 children were taken. Furthermore, we studied eight family groups of patients with resistant-*H. influenzae* meningitis (16 adults and 6 children).

The organisms were identified as *H. influenzae* by Gram stain and growth requirements of factors V and X on Mueller-Hinton agar. Isolates were typed by agglutination with specific antisera (Difco Laboratories) and biotyped by the Kilian method (10). Beta-lactamase production was detected by the rapid acidometric method (18).

Antibiotic susceptibility studies. Strains were usually tested for beta-lactamase production and for susceptibility to four antibiotics (ampicillin, chloramphenicol, tetracycline, and sulfamethoxazole-trimethoprim [SMX-TMP]) by the disk diffusion method (18). The MICs required to inhibit all CSF and blood isolates, as well as those of other sources suspected of being resistant to ampicillin and chloramphenicol, were determined by the agar dilution method. A microdilution technique (12) was also utilized simultaneously for studies of MIC with ampicillin, chloramphenicol, tetracycline, and SMX-TMP.

Disk diffusion and agar dilution testing were both performed in Mueller-Hinton agar supplemented with 5% horse blood and 1% Fildes enrichment (Difco). The inoculum was prepared by suspending growth from an overnight chocolate agar plate in Mueller-Hinton broth and adjusting the turbidity of the suspension to equal that of a 0.5 McFarland standard and then diluting the adjusted inoculum 1:10. The agar plates were inoculated by using a Steers replicator (17) delivering an inoculum of ca. 10^4 CFU onto each spot. In some experiments, the concentration of bacteria was varied

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to determine the inoculum effect. The heavy inoculum was prepared by adjusting the turbidity to match that of a no. 3 McFarland standard (ca. 9×10^9 CFU/ml), giving a final inoculum of ca. 10^6 CFU per spot. Plate counts were performed to confirm that final inocula of 10^4 and 10^6 CFU per spot were achieved. Inoculated plates were incubated at 35°C for 24 h without carbon dioxide. SMX-TMP MIC determinations were performed utilizing 10^4 CFU per spot on Mueller-Hinton agar and 10^4 CFU/ml in broth supplemented with 5% lysed horse blood and 5% Fildes enrichment (11). Mueller-Hinton broth supplemented with 5% Fildes enrichment was used in microdilution techniques.

MIC was defined as the least concentration of antimicrobial agent that permitted growth of no more than one colony within the area of inoculation (18).

Twenty-nine selected strains were sent to the Centers for Disease Control, Atlanta (C. Thornsberry), for confirmation of antimicrobial susceptibility.

Antibiotics. The antibiotic powders were laboratory standards of assayed potency and were gifts from the following pharmaceutical companies: Antibioticos S.A., Madrid, Spain (tetracycline, ampicillin, gentamicin, and rifampin), Parke, Davis & Co. (chloramphenicol), Eli Lilly & Co. (cefamandole, cephalothin, and moxalactam), Merck Sharp & Dohme (cefoxitin), Glaxo, Inc. (cefuroxime), Roche Diagnostics, Div. Hoffman-La Roche, Inc. (SMX-TMP), Hoechst-Roussel Pharmaceuticals Inc. (cefotaxime), Beecham Laboratories (carbenicillin), and Pfizer Inc. (cefoperazone).

RESULTS

Invasive diseases. During the study period, 44 *H. influenzae* strains were isolated from CSF (28) and blood (16) of an identical number of patients. MICs are shown in Table 1. A total of 22 (50%) were resistant to ampicillin, 23 (52.2%) to chloramphenicol, 24 (54.5%) to tetracycline, and 28 (63.6%) to SMX-TMP. Their resistance patterns are shown in Table 2; 20 strains (45.4%) were multiply resistant to ampicillin, chloramphenicol, and other agents; 16 came from CSF of patients with meningitis (57% of the total CSF strains), and 4 came from bacteremic patients (one epiglottitis and three pneumonias). Eighteen ampicillin- and chloramphenicol-resistant strains were also resistant to tetracycline and SMX-TMP; one was also resistant to tetracycline and one to SMX-TMP. Thirteen strains were susceptible to all four antibiotics.

Noninvasive diseases. Of 181 strains isolated from patients with noninvasive infections, 21 were found to be resistant to ampicillin and chloramphenicol. These 21 resistant strains were further studied. All were found to be resistant to tetracycline, and all but two were also SMX-TMP-resistant isolates. Seven were type b (all middle-ear exudates), and 14 were nontypable strains. MIC ranges of these strains were as follows: ampicillin, 2 to 32 μg/ml (MIC required to inhibit 90% of strains [MIC_{90}], 16 μg/ml); chloramphenicol, 8 to 32 μg/ml (MIC_{90}, 16 μg/ml); tetracycline, 8 to 32 μg/ml (MIC_{90}, 16 μg/ml); and SMX-TMP, 19-1 to 152-8 μg/ml (MIC_{90}, 76-4 μg/ml).

Carriers. Of the 22 family contacts studied from eight patients with ampicillin- and chloramphenicol-resistant *H. influenzae* meningitis, 8 were found to be colonized by *H. influenzae* strains. Three children and two adults (50 and 12.5% of total children and adults studied, respectively) were carriers of multiply resistant strains, belonging to the same serotype (b) and biotype (I) as the respective index case. The remaining three strains were susceptible, non-type b strains.

TABLE 1. Susceptibility to four antibiotics of 44 strains of *H. influenzae* isolated from CSF and blood

Antibiotic[a]	No. of isolates inhibited by the following concn (μg/ml):							
	≤0.25	0.5	1	2	4	8	16	32
Ampicillin	21	1		2	8	7	3	2
Chloramphenicol	4	17				4	16	3
Tetracycline	5	10	5			2	20	2

[a] Number of isolates inhibited by SMX-TMP and the concentration required (micrograms per milliliter): 14, ≤ 0.6 to 0.03; 2, 1.2 to 0.06; 3, 9.5 to 0.5; 9, 19 to 1; 11, 38 to 2; 5, ≥76 to 4.

Of the 103 children studied from the nurseries, 17 were colonized by *H. influenzae* type b, and 55 were colonized by non-type b strains; 31 (43%) were multiply resistant, and of them, 12 showed the same serotype (b), biotype (I), and resistance patterns as the respective index case. One belonged to type c, one belonged to type f, and the remaining strains were nontypable. No susceptibilities or resistance pattern differences were found among the three groups of carriers studied (nurseries and family carriers), and data concerning their antibiotic susceptibilities and resistance patterns are given together in Tables 3 and 4.

Other studies. All ampicillin- and chloramphenicol-resistant strains were beta-lactamase producers. The MICs obtained by agar dilution and microdilution were found to be equal or within one doubling dilution of each other. By the disk diffusion method, all resistant strains were easily identified (Table 5). For instance, all strains with MICs ≥8 μg/ml had inhibition zone diameters of ≤18 mm for chloramphenicol.

The susceptibilities to 10 additional drugs were tested on multiply resistant strains by the agar dilution method at two different inoculum sizes (Table 6). The most active drugs were cefotaxime, moxalactam, and cefoperazone, followed by cefuroxime, cefamandole, rifampin, and gentamicin. When a higher inoculum (10^6 CFU/ml) was used, an important effect was clearly seen for carbenicillin, cephalothin, and cefamandole. Inoculum effect was also apparent for ampicillin but not for chloramphenicol (data not shown).

Studies at the Centers for Disease Control (C. Thornsberry) confirmed our findings in regard to the susceptibilities of the 29 strains submitted. Discrepancies in susceptibilities to SMX-TMP were observed with three strains. By the tube dilution method, the MIC (determined at the Centers for Disease Control) required to inhibit these three strains was ≤0.6-0.03 μg/ml, and the MBC was >76-4 μg/ml. In our

TABLE 2. Antibiotic resistance patterns of 44 strains of *H. influenzae* isolated from CSF and blood

Resistance[a]	No. of resistant strains (%)
SMX-TMP	5 (11.3)
A and SMX-TMP	1 (2.2)
C and T	2 (4.5)
T and SMX-TMP	1 (2.2)
A, T, and SMX-TMP	1 (2.2)
C, T, and SMX-TMP	1 (2.2)
A, C, and SMX-TMP	1 (2.2)
A, C, and T	1 (2.2)
A, C, T, and SMX-TMP	18 (40.9)

[a] Strains resistant to ampicillin (A), chloramphenicol (C), tetracycline (T), and SMX-TMP.

TABLE 3. Susceptibility to four antibiotics of 80 strains of *H. influenzae* isolated from nasopharyngeal carriers

Antibiotic[a]	No. of isolates inhibited by the following concn (μg/ml):							
	≤0.25	0.5	1	2	4	8	16	32
Ampicillin	35	2		4	15	18	4	2
Chloramphenicol	8	34			3	11	20	4
Tetracycline	12	18	10			5	28	7

[a] Number of isolates inhibited by SMX-TMP and the concentration required (micrograms per milliliter): 25, ≤0.6 to 0.03; 6, 1.2 to 0.06; 2, 2.4 to 0.12; 4, 9.5 to 0.5; 8, 19 to 1; 18, 38 to 2; 17, ≥76 to 4.

laboratory, these strains were resistant by the disk diffusion method, and the MIC was 19-1 μg/ml.

DISCUSSION

The isolation of ampicillin- and chloramphenicol-resistant *H. influenzae* has been reported by Kenney et al. (9), Uchiyama et al. (19), MacMahon et al. (13), Heymann et al. (8), and Simasathien et al. (16) in various parts of the world. Except for the latter publication, all of the others were case reports in which the isolation of such resistant strains was an exceptional finding. All of the strains described, except for one nontypable strain (8), belonged to serotype b and had been recovered from CSF (9, 16, 19) and the respiratory tract (8, 13, 16).

The number and distribution of strains of *H. influenzae* resistant to ampicillin and chloramphenicol, as studied in this paper, constitute the most important description to date. Our strains originated just as often from nasopharyngeal carriers as from children suffering from meningitis, bacteremia, otitis, and conjunctivitis. They do not constitute a sporadic finding but a continuous problem over a prolonged period of time. No epidemiological link could be established among the cases with invasive diseases. They came from different geographic areas, and we did not notice an accumulation of cases in the period of study. Accordingly, the emergence of multiply resistant strains seems to be an endemic problem in this country. We believe that the uncontrolled and widespread use of antibiotics in Spain over many years played a role in the emergence of these multiply resistant strains, as appears to be the case with pneumococcus in the same area (15).

The problem was particularly serious in strains causing invasive infections (meningitis and bacteremia). The individual resistance to ampicillin (50%), chloramphenicol (52.2%), tetracycline (54.5%), and SMX-TMP (63.6%), as well as multiple resistance (45.4%), constitutes the most important report known of invading *H. influenzae* type b strains

TABLE 4. Antibiotic resistance patterns of 80 strains of *H. influenzae* isolated from carriers

Resistance[a]	No. of resistant strains (%)
A	2 (2.5)
SMX-TMP	4 (5)
A and SMX-TMP	3 (3.7)
A, T, and SMX-TMP	2 (2.5)
C, T, and SMX-TMP	2 (2.5)
A, C, T, and SMX-TMP	36 (45)

[a] Strains resistant to ampicillin (A), chloramphenicol (C), tetracycline (T), and SMX-TMP.

TABLE 5. Comparison of the antimicrobial activity of four antibiotics against 77 ampicillin- and chloramphenicol-resistant strains[a] of *H. influenzae*, measured by the disk diffusion method

Antibiotic	Disk concn (μg)	Inhibitory zone diam (mm)		Control group[b] diam (mm)	
		Range	Median	Range	Median
Ampicillin	10	6–18	12.8	22–34	29.2
Chloramphenicol	30	12–18	15.2	26–44	34.6
Tetracycline	30	6–17	9.9	22–32	27.6
SMX-TMP	1.25-23.75	6–16	10.8	21–33	27

[a] 76 strains were resistant to tetracycline, and 74 were resistant to SMX-TMP.

[b] Control group is susceptible strains isolated from invasive and noninvasive diseases.

resistant to first-line antibiotics. A total of 57% of CSF isolates were resistant to both ampicillin and chloramphenicol; fortunately, in our hospital *H. influenzae* is second to *N. meningitidis* in the etiology of bacterial meningitis beyond the neonatal period, with a global incidence of 10% of the cases.

In our resistant strains, the most frequent resistance pattern was ampicillin-chloramphenicol-tetracycline-SMX-TMP (94.8%), followed by ampicillin-chloramphenicol-tetracycline (3.9%) and ampicillin-chloramphenicol-SMX-TMP (1.3%). The disk diffusion method correctly predicted multiple resistance. We suggest that strains with zone diameters of ≤18 mm for chloramphenicol be regarded as probably resistant to this antibiotic. Should this be the case, the susceptibility to other alternative agents, especially new cephalosporins, becomes mandatory, particularly for the treatment of invasive diseases.

The choice of empiric antibiotic therapy should be based on knowledge of local prevalence of antibiotic resistance. Our findings clearly indicate that in our country, depending on the site of the infection, cefuroxime, cefotaxime, moxalactam, or cefoperazone should be used until the results of susceptibility testing are available. Meningitis caused by strains resistant to both ampicillin and chloramphenicol constitutes an emergency; recent publications indicate that cefuroxime (6), cefotaxime (1), moxalactam (14), and ceftriaxone (7) could constitute valid therapeutic alternatives because of their excellent activity against *H. influenzae*, whether producing beta-lactamase or not, and because of

TABLE 6. Susceptibilities to 10 drugs of 77 ampicillin- and chloramphenicol-resistant strains of *H. influenzae* measured by the agar dilution method

Antibiotic	Susceptibility with the following inoculum size (CFU per spot):			
	10^4		10^6	
	MIC_{90}	MIC range	MIC_{90}	MIC range
Carbenicillin	4	0.5–16	16	4–64
Cephalothin	8	2–8	32	4–32
Rifampin	1	0.125–1	ND[a]	
Gentamicin	1	1–2	ND	
Cefamandole	0.8	0.2–0.8	3.2	0.4–3.2
Cefoxitin	4	1–4	8	2–8
Cefuroxime	0.5	0.25–0.5	0.5	0.5
Cefoperazone	0.03	≤0.015–0.06	0.06	≤0.015–0.12
Cefotaxime	0.03	≤0.015–0.06	0.03	≤0.015–0.06
Moxalactam	0.03	≤0.015–0.06	0.06	≤0.015–0.12

[a] ND, Not done.

good penetration in the CSF of patients with bacterial meningitis.

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